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A Motility Revertant of the *ndvB* Mutant of *Bradyrhizobium japonicum*

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Abstract. A motility revertant of a *Bradyrhizobium japonicum ndvB* mutant was isolated and characterized. The *ndvB* mutants of *B. japonicum* have been reported to be osmotically sensitive, as well as defective in motility, periplasmic cyclic β-(1→3), (1→6)-D-glucan synthesis, and symbiosis with soybean. The motility revertant was restored for osmotic tolerance but not for cyclic β-glucan production or effective symbiosis. These results support our hypothesis that cyclic β-glucans have an important role in symbiosis—the suppression of a plant defense response—in addition to their role in periplasmic osmoprotection.

Microbial cells need to adapt to various environments and conditions of varying osmolarity and they have developed multiple mechanisms to function under these stresses. In the periplasm of the cell, a class of molecules named MDOs (membrane-derived oligosaccharides) were shown to function as osmolytes in *Eschericia coli* [13]. Substituted cyclic β-(1→2)-D-glucans have been found to play a similar role in many of the *Rhizobiaceae* (see review by Breedveld and Miller [6]). The cyclic glucans are small molecules that, in *Agrobacterium* and *Rhizobium,* are composed of 17–24 glucose molecules in the ring structure. The homologous *ndvB* (nodule development) and *chvB* (chromosomal virulence) genes (of *Rhizobium* and *Agrobacterium,* respectively) code for large-membrane proteins, which may be intermediates in β-(1→2)-glucan synthesis [11, 19]. The *ndvA* and *chvA* genes code for proteins presumably involved in the transport of the glucans from the cytoplasm to the periplasm [18]. Mutations in the *ndvA* and *ndvB* genes of *Sinorhizobium meliloti* cause defects in symbiotic characteristics, such as attachment to roots, infection thread formation, and nodule development. Also, they are defective in vegetative characteristics, such as osmotic tolerance, bacteriophage attachment, motility, and have an increased sensitivity to some antibiotics. These mutation-induced defects suggest that these characteristics may be due to alterations in the surface properties of the cell.

In support of this argument, symbiotic pseudorevertants of the *S. meliloti ndvB* mutation have been isolated [10] that were still unable to synthesize periplasmic β-(1→2)-glucans, but regained some symbiotic ability. Curiously, these pseudorevertants formed normal-looking nodules on approximately one in ten alfalfa plants inoculated, but continued to form uninfected nodule-like structures on the remaining plants. Quandt et al. [16] also isolated an osmorevertant of a *ndvB* mutant of *S. meliloti* and found that it still did not produce β-(1→2)-glucans, but was partially restored for infectivity. These data would suggest that β-(1→2)-glucans are not directly required for symbiotic interactions, although they play important role as periplasmic osmolytes.

In *Bradyrhizobium,* cyclic glucans are β-(1→3),(1→6)-linked and contain 10–13 glucose residues [17]. At least three genes—*ndvB, ndvC,* and *ndvD*—are required for their synthesis [1, 7]. Although the *Rhizobium* and *Bradyrhizobium* glucans are structurally different, and no homology exists between the respective structural genes, they appear to be functional analogs, at least in osmoregulation [5, 14]. In *B. japonicum,* we found that altering the structure of the glucan molecule abolished symbiotic effectiveness but did not alter osmoprotection [1]. On the other hand, a mutation

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Table 1. Symbiotic characteristics of motility revertant AB-14R2

Strain	Shoot dry weight (g/plant)[a]	Nodules per plant	Nodule fresh weight (mg/plant)	Bacteroids (CFU/nodule)[b]	Cellular glucans (μg/mg protein)[c]
USDA 110	1.59 ± 0.12	29.5 ± 2.2	1.1	$2.3 \pm 0.63 \times 10^9$	70
AB-14	0.73 ± 0.08	74.0 ± 10.5	0.55	$1.9 \pm 1.6 \times 10^6$	5
AB-14R2	0.77 ± 0.06	55.7 ± 10.2	0.65	$1.5 \pm 0.2 \times 10^6$	5
AB-14R2(p530)[d]	1.52 ± 0.05	27.7 ± 4.7	1.33	$5.9 \pm 2.5 \times 10^8$	67
AB-14(p530)[d]	1.5 ± 0.05	35.7 ± 8.3	1.31	$6.3 \pm 3.3 \times 10^8$	80

[a] Soybean plants were grown in Leonard jars as described [2]. Dry weight was determined by drying for six days at 60°C.
[b] Bacteroids were determined as colony forming units from nodules from 30-day-old plants. Nodules were surface sterilized in 1.1% sodium hypochloride for 5 min, followed by 75% ethanol wash for 3 min. After rinsing twice in sterile distilled water, nodules were macerated in an Eppendorf tube in a grinding buffer [12] and various dilutions were spread on AG agar plates [8] with appropriate antibiotics.
[c] Glucans were assayed as described [4]. Values are average of two experiments.
[d] p530 is a pLAFR3, containing 4.3-kb *Not*I-*Kpn*I fragment of p115 [3] that carries *ndvB*.

that completely abolished glucan synthesis resulted in an osmosensitive strain (AB-14, *ndvB*::Tn*5*) which still retained its ability to infect and form nodules [3, 9]. The nodule ultrastructure and pattern of nodulin synthesis were similar to the wild-type strain, but the nodules formed by AB-14 were ineffective in fixing nitrogen [9]. Our work with the soybean microsymbiont *B. japonicum* has led us to hypothesize that the cyclic β-glucans have an important and specific role in symbiosis in addition to osmoprotection and that, in soybean–*B. japonicum* symbiosis, there is specificity for the β-glucan structure in symbiosis but not for osmoprotection and motility [1, 15]. In this publication, we report the isolation of a motility revertant of *B. japonicum ndvB* mutant AB-14 [3]. The revertant also was partially restored for osmotolerance but failed to regain any effective symbiotic function.

To isolate motility and symbiotic revertants, the *ndvB* mutant AB-14 was spot inoculated onto semisolid arabinose–gluconate (AG) plates [8] supplemented with kanamycin and streptomycin (100 μg/mL each) and incubated at 28°C for 10 days. Cells from the outside edge of the swarm were used to inoculate fresh plates. This procedure was repeated ten times. When the isolates swarmed at approximately the same speed as the wild-type USDA 110, cells from the outside edge of the swarm were grown in AG broth. One portion of the culture was used to inoculate soybean plants to determine if there were spontaneous symbiotic revertants. No symbiotic revertants were found even after multiple independent attempts. Another portion of the culture was spread onto semisolid AG media containing kan and str. Colonies with increased motility were selected and purified multiple times. Finally, eight motility isolates were selected and examined for symbiotic effectiveness. All isolates remained defective in symbiotic interactions, and the motility revertant AB14-R2 was selected for further characterization (Table 1).

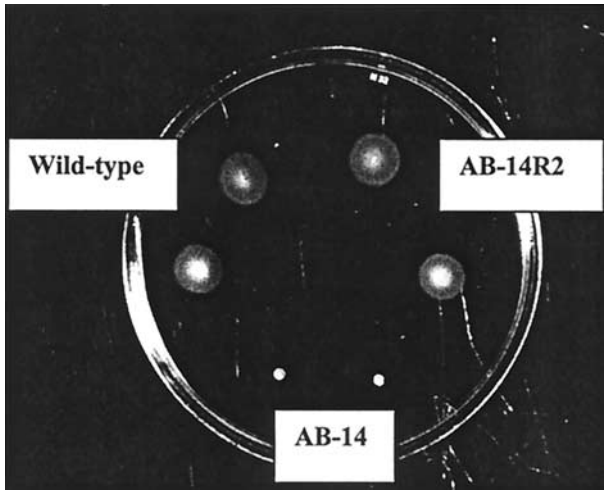

Fig. 1. Swarm formation by motility revertant AB-14R2 on soft agar. Early log phase cells of each strain were normalized to the same cell density and then spot-inoculated onto AG medium containing 0.35% agar and grown for three days at 28°C.

Figure 1 illustrates the motility revertant of AB-14. As illustrated, the swarm motility of AB-14R2 is essentially equivalent to wild-type USDA 110. Simultaneously, the revertant regained osmotolerance, as illustrated by partial restoration of growth characteristics in low osmolarity medium. The generation time of the *ndvB* mutant AB-14 was 9.1 h and of the revertant AB14R2 8.1 h, as compared with 6.8 h for the wild-type USDA 110. Also, during growth in AG medium, the lag period in AG medium of AB-14R2 was reduced significantly, as compared with AB-14 (data not shown). In spite of these

physiological gains, AB-14R2 remained defective in nodule development and infected host cells poorly. There were 1000-fold less bacteroids per nodule as compared to wild-type nodules (Table 1).

To determine the location of Tn*5* in AB-14R2 and verify that no Tn*5* excision or new transposition events occurred in AB-14R2, bacteroids were isolated from ten individual nodules inoculated with AB-14R2. Genomic DNA was isolated from the bacteroids, digested with various restriction enzymes, and used to carry out hybridization with both a digoxigenin-labeled 5.2-kb *Eco*R1 fragment containing wild-type *ndvB* and a 3.4-kb digoxigenin-labeled *Hind*III fragment of Tn*5*. These two probes were prepared by elongation of random oligonucleotides with the Klenow fragment of polymerase (DIG-High Prime Kit, Boehringer, Mannheim). An 11-kb *Eco*RI band was detected in bacteroids isolated from each of the ten nodules when probed with both the 5.2-kb probe from USDA-110 and the 3.4-kb probe from Tn*5*. Southern blot analysis indicated that the Tn*5* in AB-14R2 was located at the original site of AB-14 and that no additional transposition events occurred (data not shown).

The restorations of motility and osmotolerance were not reflected in any restoration of symbiotic function. As illustrated in Table 1, AB-14R2 did not synthesize glucans in vivo and the nodulation characteristics were similar to the parent strain, AB-14, that forms only white pseudonodules. When the parental *ndvB* mutant strain, AB-14, and the motility revertant strain, AB-14R2, were complemented with a plasmid (p530) carrying *ndvB,* symbiosis was restored to wild-type effectiveness. The greater numbers of nodules per plant that are found with AB-14 and AB-14R2, as compared with wild-type, are fairly characteristic of ineffective strains [9]. These characteristics further delineate the vegetative characteristics (motility and periplasmic osmoprotection) associated with cyclic β-(1→3),(1→6)-glucans in *B. japonicum* from their more specific role in symbiotic plant-microbe interactions. The data further supports our hypothesis that the β-(1→3),(1→6)-D-glucans of *B. japonicum* probably have an important role in symbiosis, in addition to their role in periplasmic osmoprotection.

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